THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 238, No. 4, April 1963
*Printed in U.S.A.*



# Thyroxine Stimulation of Amino Acid Incorporation into Protein

## LOCALIZATION OF STIMULATED STEP*

LOUIS SOKOLOFF, SEYMOUR KAUFMAN, PHYLLIS L. CAMPBELL, CATHERINE M. FRANCIS, AND HARRY V. GELBOIN

*From the Laboratories of Clinical Science and Cellular Pharmacology, National Institute of Health, United States Public Health Service, Bethesda, Maryland*

(Received for publication, September 10, 1962)

Previous studies in this laboratory have demonstrated that the prior administration of L-thyroxine to animals *in vivo* or its addition *in vitro* stimulates the rate of amino acid incorporation into microsomal protein in cell-free rat liver homogenates (3). Mitochondria and an oxidizable substrate were found to be essential requirements for the thyroxine effect *in vitro*; when these components of the system were replaced by a creatine phosphate-adenosine triphosphate-generating system, no thyroxine effects were observed.

Current concepts of protein biosynthesis support the following abbreviated scheme for the incorporation of amino acids (AA) into microsomal protein (4, 5).[1]

$$\text{AA} + \text{ATP} \xrightleftharpoons{\text{AA-activating enzyme}} \text{AA-AMP-enzyme} + \text{PP}_i \quad (1)$$

$$\text{AA-AMP-enzyme} + \text{sRNA} \rightleftharpoons \text{sRNA-AA} + \text{AMP} + \text{enzyme} \quad (2)$$

$$\text{sRNA-AA} + \text{microsomes} \xrightarrow{\text{GTP}} \text{protein-AA} + \text{sRNA} \quad (3)$$

The present studies demonstrate that the thyroxine effect on amino acid incorporation into protein is entirely the result of a stimulation of the last step in the reaction sequence above. The thyroxine stimulation of the soluble RNA-bound amino acid transfer to microsomal protein has been found to have the same requirements and characteristics as the effect on free amino acid incorporation.

### EXPERIMENTAL PROCEDURE

#### *Materials*

*Chemicals*—Compounds obtained from commercial sources were of the highest grade of purity available. Uniformly labeled L-valine-$C^{14}$ (specific activity = 6.51 mc per mmole) and DL-valine-1-$C^{14}$ (specific activity = 3.05 mc per mmole) were obtained from the Nuclear-Chicago Corporation. L-Valine-1-$C^{14}$ (specific activity = 12.0 mc per mmole) was purchased from the California Corporation for Biochemical Research. Nonisotopic L-valine was obtained from Schwarz BioResearch, Inc. GTP was purchased from Pabst Laboratories and Sigma Chemical Company. Glutathione, oxidized and reduced, was obtained from the California Corporation for Biochemical Research.

Soluble ribonucleic acid was prepared from rabbit liver by the NaCl extraction method of Cantoni *et al.* (6). Uniformly labeled soluble RNA-L-valine-$C^{14}$ and soluble RNA-L-valine-1-$C^{14}$ were prepared by incubating the soluble RNA with the appropriate L-valine-$C^{14}$ and ATP in the presence of partially purified beef liver L-valine activating enzyme and other necessary cofactors, and were then extracted from the incubation mixture and purified according to the methods of Cantoni *et al.* (6, 7). Although the soluble RNA-L-valine-$C^{14}$ preparations appeared to be stable for at least 3 months at −20°, they were, nevertheless, dialyzed against a minimum of 400 volumes of a solution of 0.1 M NaCl-0.005 M potassium phosphate buffer, pH 7.4, at 0–4° for 4 hours immediately before use, and aliquots of the dialyzed solutions were assayed for their RNA and radioactive amino acid concentrations as described below.

All other compounds and enzymes were the same as those previously described (3).

*Animals*—Normal Sprague-Dawley or Osborne-Mendel male rats weighing between 70 and 120 g were used in all experiments. Similar results were obtained with both strains. Animals were maintained on Purina laboratory chow fed *ad libitum* but were deprived of food for at least 17 hours before killing.

#### *Methods*

*Preparation of Homogenates*—Liver homogenates were prepared fresh for each experiment. Homogenization procedures were the same as those previously described by Sokoloff and Kaufman (3). For experiments in which all flasks contained the complete system, *i.e.* mitochondria, microsomes, and cell sap, fractionation and reconstitution of the crude homogenate were carried out in accordance with the procedure designated by them as Procedure A. In those experiments in which thyroxine effects in the complete system were compared with those obtained when the mitochondrial oxidative phosphorylation system was replaced by a creatine phosphate-ATP-generating system (Table II), their Procedure C was employed.

*Incubation*—Incubation procedures were the same as those previously described (3). The components of the incubation

* Preliminary reports of portions of this work have been previously presented (1, 2).

[1] The abbreviation used in the scheme is: sRNA, *soluble* or amino acid acceptor ribonucleic acid.

mixtures and the specific incubation conditions are described in the legends to the tables and figures.

*Purification and Counting of Protein Samples*—Except when specified otherwise in the legends to the tables and figures, reactions were terminated by precipitation of the protein with an equal volume of 12% trichloroacetic acid. The precipitated protein was purified, plated, and counted as previously described (3).

*Extraction, Purification, and Assay of Soluble RNA-L-Valine-$C^{14}$*—In experiments of the type illustrated in Fig. 1, incorporation of L-valine-$C^{14}$ into both soluble RNA-L-valine-$C^{14}$ and protein were measured simultaneously in the same flasks. In these experiments the reaction was stopped by the addition of 2 volumes of ice-cold 0.25 M sucrose solution containing 2 mg of nonisotopic L-valine per ml. The mitochondria were then removed by centrifugation at 12,800 × *g* for 10 minutes in the Servall refrigerated centrifuge. The 12,800 × *g* supernatant fraction was then centrifuged for 1 hour at 100,000 × *g* in the Spinco model L ultracentrifuge to separate the microsomes containing the labeled protein and the soluble fraction containing the soluble RNA-L-valine-$C^{14}$. The microsomal pellet was resuspended in 5 ml of 0.25 M sucrose solution and precipitated by the addition of an equal volume of 12% trichloroacetic acid. The protein in this fraction was then purified, plated, and counted in the usual manner as described above.

The soluble RNA-L-valine-$C^{14}$ was extracted as follows. An equal volume of ice-cold 12% trichloroacetic acid was added to the 100,000 × *g* supernatant solution, and the suspensions were allowed to sit in ice for 15 minutes to allow time for complete precipitation. The suspensions were then centrifuged for 5 minutes at approximately 2000 × *g* in the refrigerated centrifuge, the supernatant solution was decanted, and the precipitate was thoroughly resuspended in 10 ml of ice-cold 3% perchloric acid. The precipitate was sedimented again by centrifugation at 2000 × *g*, washed twice more as above in 3% perchloric acid, and then twice in 5 ml of ice-cold 0.06% perchloric acid. The supernatant solution from the centrifugation after the final 0.06% perchloric acid wash was decanted and drained completely by inversion of the tubes. The soluble RNA-L-valine-$C^{14}$ contained in the sediment was then extracted into 1 to 2 ml of an ice-cold solution of 0.1 M NaCl, 0.05 M potassium phosphate, pH 7.2. The residual protein precipitate was removed by centrifugation. The average recovery of the added soluble RNA in the soluble RNA-amino acid extract was approximately 55%.

Soluble RNA concentration in the soluble RNA-L-valine-$C^{14}$ solutions was assayed by measurement of the absorbancy of an appropriately diluted aliquot at 260 mμ, assuming an absorbancy index for rabbit liver soluble RNA of 23.0 per mg per ml for a 1-cm optical path length (8). Purity of the solutions was checked by determination of the ratio of absorbancies at 280 and 260 mμ. One milliliter of the diluted aliquot used for spectrophotometric measurement was mixed with 10 ml of a naphthalene-dioxane phosphor solution (9) and counted in the Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency was determined individually in each vial by means of $C^{14}$ internal standards which permitted conversion of the counting rates to millimicrocuries. Specific activity of the soluble RNA-L-valine-$C^{14}$, *i.e.* millimicrocuries of L-valine-$C^{14}$ per mg of soluble RNA, was calculated from the optical density and counting data. Evidence that the radioactivity in the samples was in the form of soluble RNA-L-valine-$C^{14}$ was obtained from tests

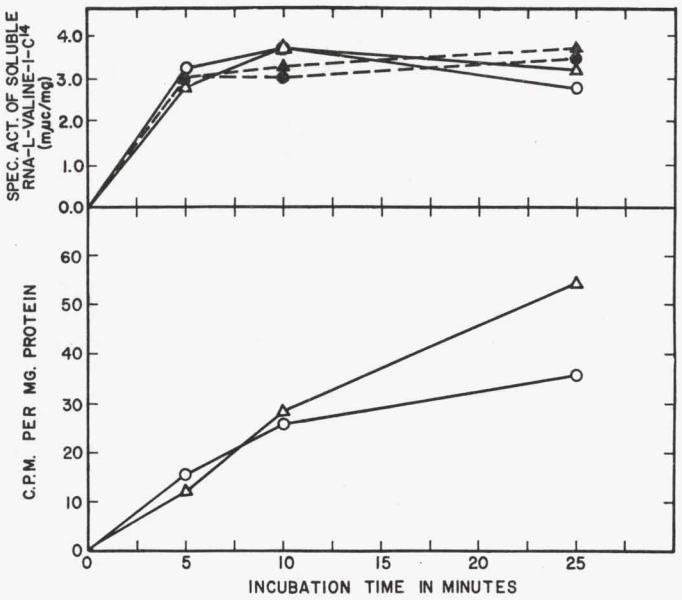


FIG. 1. Comparative effects of $6.5 \times 10^{-5}$ M L-thyroxine on the time course of incorporation of DL-valine-1-$C^{14}$ into soluble RNA-amino acid and into microsomal protein. The assay conditions were the same as those in Table I, except that all the valine-$C^{14}$ was added in the form of DL-valine-1-$C^{14}$, 0.76 μmoles per flask (specific activity = 3.05 μc per μmole), and soluble RNA was added to each of the flasks as described below. ○——○, 0.75 mg of soluble RNA, control; △——△, 0.75 mg of soluble RNA, $6.5 \times 10^{-5}$ M L-thyroxine; ●- - -●, 1.50 mg of soluble RNA, control; ▲- - -▲, 1.50 mg of soluble RNA, $6.5 \times 10^{-5}$ M L-thyroxine. The curves for the incorporation into protein are those for the flasks containing the low amounts of added soluble RNA; those for the flasks with the higher amounts of soluble RNA are identical in form but exhibit a lower specific activity. Incubation times at 37° are as indicated. The methods for the separation, purification, and assay of the specific activities of the protein and the soluble RNA-L-valine-1-$C^{14}$ are described in the text.

of the ability of a dialyzed solution of the compound to transfer its radioactivity to microsomal protein in the standard soluble RNA-bound amino acid transfer assay system.

Soluble RNA-L-valine-$C^{14}$ synthesized in the completely soluble system with the purified beef liver L-valine activating enzyme as described above was assayed for its soluble RNA and radioactivity contents similarly. Since this system contained relatively little amino acid-containing material other than the L-valine-$C^{14}$ added as substrate, the radioactivity incorporated into the soluble RNA-L-valine-$C^{14}$ was assumed to be of the same form and specific activity as the amino acid precursor; molar concentration of the soluble RNA-bound L-valine-$C^{14}$ was then calculated from the concentration of radioactivity and the specific activity of the precursor amino acid. These data were employed only for the estimation of the molar quantities of L-valine-$C^{14}$ contained in the aliquots of soluble RNA-L-valine-$C^{14}$ added to the reaction mixtures. Inasmuch as some endogenous nonradioactive L-valine may have been present, these estimates must be regarded as the lower limits.

## RESULTS

*Effects of L-Thyroxine on Transfer of Soluble RNA-Bound L-Valine-$C^{14}$ into Protein*—Because the rate of free amino acid incorporation into microsomal protein in the system employed

TABLE I

*Comparative effects of L-thyroxine on the incorporation of free and soluble RNA-bound L-valine-U-C¹⁴* into protein*

The components of the reaction mixture, in micromoles, were as follows: sucrose, 150; potassium phosphate buffer, pH 7.4, 20; $MgCl_2$, 5; AMP, 5; sodium DL-β-hydroxybutyrate, 50. In addition, each flask received 0.45 ml of homogenate prepared by Procedure A of Sokoloff and Kaufman (3), which contained mitochondria and microsomes equivalent to the yield from 200 mg and supernatant fluid equivalent to the yield from 30 mg of fresh liver. Nonradioactive L-valine, free L-valine-U-C¹⁴ (specific activity = 6.51 μc per μmole), or soluble RNA-bound L-valine-U-C¹⁴ (containing 0.37 mμmole of L-valine-U-C¹⁴ of the same specific activity per 1 mg of soluble RNA) were added as indicated. Experimental flasks received sufficient sodium L-thyroxine dissolved in 0.1 ml of 0.01 M NaOH to achieve a final concentration of $6.5 \times 10^{-5}$ M; control flasks received equivalent amounts of NaOH alone. The reaction mixture was brought to a final volume of 1.7 ml with water. Incubation time at 37° was 25 minutes. All other incubation procedures were as previously described (3).

| L-Valine-U-C¹⁴ addition | | Nonradioactive L-valine addition | Control | +L-Thyroxine | L-Thyroxine effect | |
|---|---|---|---|---|---|---|
| Form | Quantity | | | | | |
| | *mμmoles/flask* | *mμmoles/flask* | *c.p.m./mg protein* | | *Δc.p.m./mg* | *%* |
| L-Valine-U-C¹⁴ | 380 | 0 | 23.8 | 29.6 | +5.8 | +24 |
| L-Valine-U-C¹⁴ | 0.15 | 0 | 0.3 | 0.2 | | |
| Soluble RNA-bound L-Valine-U-C¹⁴ | 0.13 | 0 | 13.6 | 17.7 | +4.1 | +30 |
| Soluble RNA-bound L-Valine-U-C¹⁴ | 0.13 | 5 | 12.2 | 16.5 | +4.3 | +35 |
| Soluble RNA-bound L-Valine-U-C¹⁴ | 0.13 | 850 | 12.5 | 17.1 | +4.6 | +39 |

* L-Valine-U-C¹⁴ refers to the uniformly or randomly labeled compound and is used in the tables as an abbreviation.

here was stimulated by the addition of small amounts of GTP or increased amounts of microsomes but was relatively insensitive to changes in the mitochondrial and cell sap contents of the reaction mixture, it was conjectured that Reaction 3 in the scheme outlined above was rate limiting. The initial experiments on the localization of the thyroxine stimulation were, therefore, directed at the effects of the hormone on the transfer of the soluble RNA-bound amino acid into protein. The results of a representative experiment are presented in Table I. It can be seen that $6.5 \times 10^{-5}$ M L-thyroxine stimulates the incorporation of uniformly labeled soluble RNA-bound L-valine-C¹⁴ into protein at least as effectively as that of the free amino acid. Approximately equivalent amounts of uniformly labeled L-valine-C¹⁴ added in the free rather than the soluble RNA-bound form result in virtually unmeasurable incorporation into protein. The addition of nonradioactive L-valine in sufficient amounts to dilute the uniformly labeled soluble RNA-bound L-valine-C¹⁴ as much as 6000-fold has negligible effects on both the base-line incorporation rate and the thyroxine effect. These results indicate that the thyroxine stimulation is in the direct pathway (Reaction 3) and not on some possible alternative pathway for incorporating amino acids released from the soluble RNA-bound form by the reversibility of the preceding two reactions.

*L-Thyroxine Effect on Amino Acid Incorporation into Soluble RNA-Amino Acid*—Certain aspects of the results summarized in Table I suggested that the thyroxine effect is localized to the transfer step and does not reflect a stimulation of amino acid activation as well. First of all, the per cent stimulation by thyroxine observed with the soluble RNA-bound form is at least as great and usually greater than occurs with the free amino acid and, therefore, sufficient to account for the over-all effect. Secondly, the per cent thyroxine effect on the transfer step is not reduced by addition of the nonradioactive L-valine pool although simultaneous stimulation of amino acid activation might be expected to result in dilution of the specific activity of the uniformly labeled soluble RNA-L-valine-C¹⁴ and, therefore, reduced incorporation of the C¹⁴-labeled species.

Although suggestive, however, these experiments did not directly and conclusively exclude the possibility of some contribution to the thyroxine effect on free amino acid incorporation into protein from a simultaneous stimulation of amino acid activation or incorporation into soluble RNA-amino acid. Even if the transfer step (Reaction 3) were the rate-limiting step, in the presence of an endogenous pool of nonradioactive soluble RNA-amino acid stimulation of C¹⁴-labeled amino acid incorporation into the aminoacyl-soluble RNA pool would raise the specific activity of the soluble RNA-bound form more rapidly and, therefore, result in an increased rate of incorporation of the radioactive species into protein. This question was considered important, for the implications concerning the mechanism of the thyroxine effect would be quite different were thyroxine to stimulate both the incorporation of amino acids into soluble RNA-amino acid and the transfer step rather than the transfer step alone. Experiments were, therefore, carried out in which the effects of thyroxine on L-valine-C¹⁴ incorporation into both soluble RNA-amino acid and protein were compared simultaneously in the same flasks. In the experiment illustrated in Fig. 1, varying incubation times and different amounts of added soluble RNA were employed in order to ascertain when equilibrium between the free and soluble RNA-bound L-valine-C¹⁴ had been achieved. It can be seen that the incorporation of the amino acid into the soluble RNA-bound form very rapidly reaches equilibrium, in fact during the lag period preceding the appearance of the thyroxine effect on the incorporation into protein. This lag period has previously been reported and discussed (3). No thyroxine effects on the incorporation of amino acid into the soluble RNA-bound form were observed in these experiments. It is apparent, therefore, that the thyroxine stimulation of the free amino acid incorporation into protein is not to any measurable extent derived from an increased incorporation of amino acid into soluble RNA-amino acid and must, therefore, be localized at the transfer step (Reaction 3).

*Mitochondrial Requirement for Thyroxine Stimulation of Transfer Step*—The thyroxine stimulation of free amino acid incorporation into protein is dependent on the presence of mitochondria and an oxidizable substrate; when these components of the reaction mixture are replaced by a creatine phosphate-ATP-generating system, no thyroxine effect is observed (3). As can be seen from the results of the experiment summarized in Table II, the thyroxine stimulation of the transfer of soluble RNA-bound amino acid to microsomal protein has the same requirement. Although in the experiment in Table II the requirements for mitochondria and the oxidizable substrate were not determined separately, it is clear from other experiments that without

TABLE II

*Requirement of mitochondria and oxidizable substrate for L-thyroxine effect on transfer of soluble RNA-bound L-valine-1-$C^{14}$ to protein*

Homogenate fractions were prepared by Procedure C of Sokoloff and Kaufman (3). The complete system contained the same components as the reaction mixtures with the $C^{14}$-aminoacyl-soluble RNA described in Table I, including 0.15 ml of the mitochondrial suspension and 0.30 ml of the microsomal-supernatant mixture added separately. Each flask received 0.60 mg of soluble RNA-L-valine-1-$C^{14}$ containing 0.64 mμmoles of L-valine-1-$C^{14}$ (specific activity = 12.0 μc per μmole) per 1 mg of soluble RNA. The contents of the flasks without mitochondria and DL-β-hydroxybutyrate were identical, except that the mitochondrial suspension was replaced by 0.15 ml of 0.25 M sucrose solution, and the DL-β-hydroxybutyrate was replaced by 40 μmoles of creatine phosphate and 0.25 mg of creatine phosphokinase contained in an equivalent volume. L-Thyroxine concentration in the experimental flasks was $6.5 \times 10^{-5}$ M. Incubation time at 37° was 25 minutes. At the end of the incubation the reaction was stopped by the addition of 10 ml of 0.25 M ice-cold sucrose solution. The mitochondria were removed from the mixtures containing the complete system by 10 minutes of centrifugation at 8000 × *g* in the Servall refrigerated centrifuge. The protein in the 8000 × *g* supernatant fluids from these flasks and in the suspensions from the flasks without mitochondria were then precipitated by the addition of equal volumes of 12% trichloroacetic acid and purified and counted in the usual manner.

| System | Control | +L-Thyroxine | L-Thyroxine effect | |
|---|---|---|---|---|
| | *c.p.m./mg protein* | | *Δc.p.m./mg* | *%* |
| Complete | 16.8 | 32.7 | +15.9 | +95 |
| Minus mitochondria, minus DL-β-hydroxybutyrate, plus creatine phosphate, plus creatine phosphokinase | 20.6 | 18.7 | −1.9 | −9 |

mitochondria oxidizable substrate is insufficient for the thyroxine effect. In the presence of the mitochondrial system without added oxidizable substrate variable results are obtained; thyroxine effects may or may not be observed although, when present, they are generally less than those observed in the presence of added substrate. It is likely that this variability reflects the amounts of endogenous substrate present in the homogenate.

*Lag Period Preceding Thyroxine Effect*—The thyroxine stimulation *in vitro* of free amino acid incorporation into protein is characterized by a 5- to 7-minute lag period preceding the stimulation (Fig. 1) (3). In Fig. 2 it can be seen that the thyroxine stimulation of the transfer of soluble RNA-bound amino acid to microsomal protein exhibits the same lag. From the time course illustrated in Fig. 2, it is impossible to determine whether the thyroxine effect is preservative or stimulatory because the rate of incorporation rapidly declines from the initial rate as it becomes limited by the available soluble RNA-amino acid. However, it was previously shown (3) that when soluble RNA-amino acid is continuously regenerated, as is the situation when a large excess of free amino acid is used as the radioactive precursor, the rate of amino acid incorporation remains constant for longer periods of time; it is then apparent that the thyroxine effect is a true stimulation of the initial rate.

*Effects of GTP*—GTP appears to be the only essential nucleotide requirement for the transfer of soluble RNA-bound amino acid to protein (10, 11). Because of the requirements of mitochondria and an oxidizable substrate for the thyroxine stimulation of this transfer step, the possibility was considered that the thyroxine effect might be mediated via an effect on GTP generation. Such does not appear to be the case. As can be seen in Table III, GTP does indeed cause a small increase in the incorporation of soluble RNA-bound amino acid in the system employed in these studies, but it does not replace the thyroxine effect which remains essentially unchanged in the presence of optimal or even greater than optimal amounts of added GTP.

*Effects of ATP*—The role of ATP in the transfer of soluble RNA-bound amino acid to microsomal protein remains uncertain. It is generally believed that it is required only to regenerate the essential nucleotide, GTP (10). The suggestion has also been made that ATP may be required for the transport of the amino acid across the microsomal lipoprotein membrane (5). Because of the dependence of the thyroxine effect on

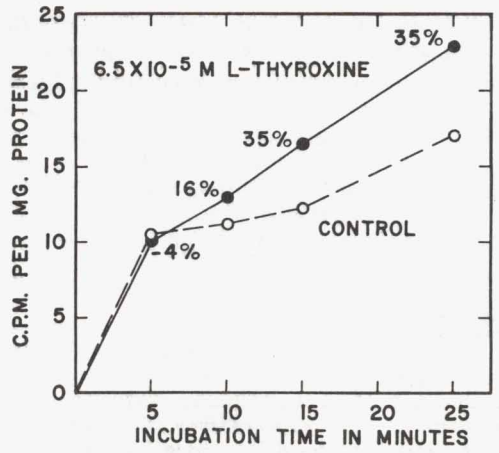


FIG. 2. Time course of the effect of $6.5 \times 10^{-5}$ M L-thyroxine on the transfer of soluble RNA-bound L-valine-1-$C^{14}$ into protein. The assay conditions were the same as those in Table I, except that all the flasks received the L-valine-$C^{14}$ in the form of soluble RNA-bound L-valine-1-$C^{14}$, 0.72 mg per flask, containing 0.62 mμmoles of L-valine-1-$C^{14}$ (specific activity = 12.0 μc per μmole) per mg. Incubation time at 37° was as indicated. The per cent L-thyroxine effects are indicated directly on the graph.

TABLE III

*Effects of GTP on L-thyroxine stimulation of soluble RNA-bound L-valine-U-$C^{14}$ incorporation into protein*

The assay conditions were the same as those described in Table I, except that all the L-valine-U-$C^{14}$ was added in the form of soluble RNA-L-valine-U-$C^{14}$, 0.53 mg per flask containing 0.92 mμmole of L-valine-U-$C^{14}$ (specific activity = 6.51 μc per μmole) per 1 mg of soluble RNA. Each flask received the amounts of GTP indicated. Final L-thyroxine concentration in the experimental flasks was $6.5 \times 10^{-5}$ M. Incubation time at 37° was 25 minutes.

| GTP addition | Control | +L-Thyroxine | L-Thyroxine effect | |
|---|---|---|---|---|
| *μmoles/flask* | *c.p.m./mg protein* | | *Δc.p.m./mg* | *%* |
| None | 15.2 | 20.3 | +5.1 | +34 |
| 0.25 | 18.3 | 24.8 | +6.5 | +36 |
| 0.50 | 16.0 | 23.6 | +7.6 | +48 |
| 0.75 | 15.3 | 21.7 | +6.4 | +42 |

mitochondria and an oxidizable substrate and the findings by Bronk (12) that thyroxine may under certain conditions stimulate oxidative phosphorylation, studies were carried out to examine the possibility that the thyroxine effect on amino acid incorporation was secondary to an effect on ATP generation. The results of a representative experiment are summarized in Table IV. Total adenine nucleotide concentration was kept constant, but the proportions of AMP and ATP were varied to simulate the effects of oxidative phosphorylation. It can be seen that with increasing proportions of ATP, there is a progressive decline in the transfer of soluble RNA-bound amino acid to protein, indicating that the ATP generation in the standard system is more than adequate. The thyroxine effect remains, however, essentially unchanged, at least until the incorporation is reduced to very low levels by the inhibitory effect of the ATP. Increased ATP does not replace the thyroxine effect as it might be expected to do if the thyroxine effect resulted from a stimulation of ATP generation, nor does the thyroxine effect become greater with greater inhibition by more ATP as might be expected if the thyroxine effect were secondary to reduced ATP generation. The inhibitory effect of the added ATP is not the result of the additional phosphate added with it; equivalent amounts of inorganic phosphate have negligible effects on the transfer step. $Mg^{++}$ binding may, however, be involved, for the ATP inhibition may be almost entirely overcome by increasing the $Mg^{++}$ concentration. It appears, therefore, that the thyroxine effect on the incorporation of soluble RNA-bound amino acid is not the result of an effect on ATP generation.

TABLE IV

*Effects of ATP on L-thyroxine stimulation of soluble RNA-bound L-valine-1-$C^{14}$ incorporation into protein*

The assay conditions were the same as those in Table I, except that all the L-valine-$C^{14}$ was added in the form of soluble RNA-L-valine-1-$C^{14}$, 0.52 mg per flask containing 0.64 mμmole of L-valine-1-$C^{14}$ (specific activity = 12.0 μc per μmole) per 1 mg of soluble RNA, and the AMP and ATP additions per flask were as indicated in the Table. Final L-thyroxine concentration in the experimental flasks was $6.5 \times 10^{-5}$ M. Incubation time at 37° was 25 minutes.

| Adenine nucleotide addition | | Control | +L-Thyroxine | L-Thyroxine effect | |
|---|---|---|---|---|---|
| AMP | ATP | | | | |
| *μmoles/flask* | | *c.p.m./mg protein* | | *Δc.p.m./mg* | *%* |
| 5.00 | 0.00 | 14.9 | 21.8 | +6.9 | +46 |
| 3.75 | 1.25 | 10.9 | 16.0 | +5.1 | +47 |
| 2.50 | 2.50 | 6.6 | 9.5 | +2.9 | +44 |
| 0.00 | 5.00 | 3.9 | 4.6 | +0.7 | +18 |

TABLE V

*Effects of GSH on the L-thyroxine stimulation of soluble RNA-bound L-valine-1-$C^{14}$ incorporation into protein**

The assay conditions were the same as those in Table I, except that each flask contained 0.25 μmole of GTP, and the L-valine-$C^{14}$ was added in the form of soluble RNA-L-valine-1-$C^{14}$, 0.84 mg per flask containing 0.64 mμmole of L-valine-1-$C^{14}$ (specific activity = 12.0 μc per μmole) per 1 mg of soluble RNA. Final L-thyroxine concentration in the experimental flasks was $6.5 \times 10^{-5}$ M. Incubation time at 37° was 25 minutes.

| GSH addition | Control | +L-Thyroxine | L-Thyroxine effect | |
|---|---|---|---|---|
| *μmoles/flask* | *c.p.m./mg protein* | | *Δc.p.m./mg* | *%* |
| 0 | 48.8 | 74.0 | +25.2 | +52 |
| 30 | 78.5 | 114.7 | +36.2 | +46 |
| 60 | 77.1 | 110.0 | +32.9 | +43 |
| 90 | 87.1 | 109.9 | +22.8 | +26 |
| 120 | 85.5 | 103.0 | +17.5 | +20 |

* The radioactivity data in this experiment were obtained with the Tracerlab model No. FD1-P1 window-flow counter which had almost twice the counting efficiency as the counter used in the experiments summarized in the previous tables and figures.

*Effect of GSH*—GSH, or some other sulfhydryl compound, has been reported to stimulate or to be a requirement in the transfer of amino acids from aminoacyl-soluble RNA into protein in mammalian systems (13, 14). In the system employed in these studies, marked stimulation of the transfer step by GSH was observed (Table V). Oxidized glutathione inhibited markedly. Although the optimal GSH concentration appeared to be lower in the presence of thyroxine than in its absence, the thyroxine stimulation of the transfer step was still superimposed on the effects of even saturating amounts of GSH, indicating that the thyroxine effect was probably not mediated by an effect on the GSH level of the system.

## DISCUSSION

The results of the present studies demonstrate that the thyroxine stimulation of amino acid incorporation into protein is localized at the step involving the transfer of soluble RNA-bound amino acid into microsomal protein. The specific characteristics of the stimulation of the transfer step are the same as those of the effect on free amino acid incorporation into protein, for example, the requirement of mitochondria and an oxidizable substrate and the short lag period preceding the stimulation. It is, therefore, reasonable to assume that the other properties of the thyroxine effect described previously in relation to the stimulation of free amino acid incorporation into protein (3) also apply to the effect on the transfer step.

Although the mechanism of the thyroxine effect remains obscure, certain possible explanations appear to have been excluded. Of the various known or suspected cofactor requirements for the transfer step, GTP, ATP, and GSH, none appears to substitute for thyroxine. The thyroxine stimulation is present essentially unaltered in the presence of saturating amounts of each, indicating that it is not secondary to an effect on the generation of these cofactors.

The dependence of the thyroxine effect on the presence of mitochondria and an oxidizable substrate presents a perplexing but intriguing implication concerning the mechanism of the effect. There is no evidence of any role for mitochondria in the transfer of soluble RNA-bound amino acid to microsomal protein, except to provide an ATP-generating system to regenerate the essential nucleotide, GTP. Substitution of a creatine phosphate-ATP-generating system for the mitochondrial system adequately maintains the rate of the transfer step but is insufficient as regards the thyroxine effect. Clearly the mitochondrial system plays some role in the mechanism of the thyroxine effect. Further implications concerning the nature of the role of the mitochondria may be derived from the 5- to 7-minute lag period preceding the thyroxine effect on amino acid incorporation into protein (Figs. 1 and 2). The characteristics of this lag have been described in detail previously (3). The lag period can be elimi-

nated by a short preincubation at 37°, provided thyroxine and the oxidizable substrate are both present during the preincubation; if either is absent during the preincubation, the lag period remains unaltered. Recent experiments have demonstrated that the microsomal system need not be present during the preincubation for the elimination of the lag in the thyroxine effect.[2] Preliminary studies also indicate that the thyroxine effect can be transferred by the addition of the soluble supernatant fractions from the preincubated mitochondrial system to a microsomal system for incorporating soluble RNA-bound amino acid into protein in which the direct addition of thyroxine is ineffective.[2] These results suggest that a preliminary or intermediate interaction between thyroxine and the mitochondrial system precedes the stimulation of the transfer of soluble RNA-bound amino acid into microsomal protein. The mechanism of the thyroxine effect appears, therefore, to resolve itself into two problems. First, there are the questions concerning the nature and the products of the preliminary interaction between thyroxine and the mitochondrial system, whether there is a stimulating substance produced or inhibition of the formation of an inhibitor. Is the active intermediate a product of thyroxine or a mitochondrial product induced or released by thyroxine? Thyroxine has been reported by Lehninger *et al.* (15, 16) to release a substance, designated by them as U-factor, which alters mitochondrial permeability; conceivably it or some similar substance might also alter microsomal permeability to soluble RNA-bound amino acid. Secondly, there is the question of the mechanism of the stimulation of the transfer step. Is it a matter of increased microsomal permeability or the acceleration of one of the many intermediate reactions involved in this step? These questions are currently under investigation.

## SUMMARY

1. Studies on the mechanism of the L-thyroxine stimulation of amino acid incorporation into protein in a cell-free rat liver system have localized the stimulation to the step involving the transfer of soluble ribonucleic acid-bound amino acid to microsomal protein. The L-thyroxine effect on the transfer step is at least as great or greater than that on the incorporation of the free amino acid. Incorporation of amino acid into aminoacyl-soluble ribonucleic acid rapidly reaches equilibrium, in fact before the end of the 5-minute lag period preceding the L-thyroxine effect on amino acid incorporation into microsomal protein; no L-thyroxine effects on amino acid incorporation into aminoacyl-soluble ribonucleic acid were observed.

2. The L-thyroxine effect on the transfer step exhibits the same characteristics and dependencies as the effect on the incorporation of the free amino acid, for example, a 5-minute lag period preceding the appearance of the effect on amino acid incorporation into protein and a requirement for mitochondria and an oxidizable substrate. When these two components of the system are replaced by a creatine phosphate-adenosine triphosphate-generating system, no thyroxine effects are observed.

3. The L-thyroxine stimulation of the transfer step is observed in the presence of optimal or greater than optimal amounts of guanosine triphosphate, the only known essential nucleotide requirement for this step. The per cent thyroxine effect remains essentially unchanged by the addition of varying amounts of adenosine triphosphate which inhibit the transfer step to varying degrees. Reduced glutathione stimulates the transfer reaction markedly, but the thryoxine effect, although somewhat reduced in degree, is still observed in the presence of the relatively high concentrations of reduced glutathione required for saturation. These results suggest that the thyroxine stimulation of the transfer step is not secondary to an effect on guanosine triphosphate, adenosine triphosphate, or reduced glutathione generation.

*Acknowledgments*—The authors wish to express their gratitude to Miss Sydell Gelber, Mr. John Cason, and Mr. Harold McCann for their skillful technical assistance.

[2] L. Sokoloff, P. L. Campbell, and C. M. Francis, unpublished observations.

## REFERENCES

1. Sokoloff, L., Kaufman, S., and Gelboin, H. V., *Biochim. et Biophys. Acta*, **52**, 410 (1961).
2. Sokoloff, L., Campbell, P. L., Francis, C. M., and Gelboin, H. V., *Federation Proc.*, **21**, 217 (1962).
3. Sokoloff, L., and Kaufman, S., *J. Biol. Chem.*, **236**, 795 (1961).
4. Zamecnik, P. C., in *The Harvey lectures, 1958–1959*, Academic Press, Inc., New York, 1960, p. 256.
5. Hoagland, M. B., in E. Chargaff and J. N. Davidson (Editors), *The nucleic acids, Vol. III*, Academic Press, Inc., New York, 1960, p. 349.
6. Cantoni, G. L., Gelboin, H. V., Luborsky, S. W., Richards, H. H., and Singer, M. F., *Biochim. et Biophys. Acta*, **61**, 354 (1962).
7. Klee, W., and Cantoni, G. L., *Proc. Natl. Acad. Sci. U. S.*, **46**, 322 (1960).
8. Luborsky, S. W., and Cantoni, G. L., *Biochim. et Biophys. Acta*, **61**, 481 (1962).
9. Werbin, H., Chaikoff, I. L., and Imada, M. R., *Proc. Soc. Exptl. Biol. Med.*, **102**, 8 (1959).
10. Grossi, L. G., and Moldave, K., *J. Biol. Chem.*, **235**, 2370 (1960).
11. Nathans, D., von Ehrenstein, G., Monro, R., and Lipmann, F., *Federation Proc.*, **21**, 127 (1962).
12. Bronk, J. R., *Biochim. et Biophys. Acta*, **37**, 327 (1960).
13. Hülsmann, W. C., and Lipmann, F., *Biochim. et Biophys. Acta*, **43**, 123 (1960).
14. Bishop, J. O., and Schweet, R. S., *Biochim. et Biophys. Acta*, **49**, 235 (1961).
15. Lehninger, A. L., and Remmert, L. F., *J. Biol. Chem.*, **234**, 2459 (1959).
16. Wojtczak, L., and Lehninger, A. L., *Biochim. et Biophys. Acta*, **51**, 442 (1961).

NIH 28468